Abstract
Structural solutions adapted by proteins to withstand changes in temperature have basic importance in the understanding of protein structure-function relations and also in industrial enzymology. Adaptability of protein structure is exemplified by the discovery of enzymes that function at extremes of environment. Proteins from extremophiles have become important in deciphering the strategies adapted by proteins to retain their functionalities in extremes of environment. The conventional approach taken to understand thermostability in proteins is to compare the structures of homologous proteins isolated from thermophiles, mesophiles and psychrophiles. Such approaches have yielded insights into the importance of hydrophobicity, surface loops, salt bridges, packing interactions etc. in protein thermostability. Given that the enzymes have evolved over long periods during which thermal stress might not have been the only condition the protein had to adapt to, information gathered by this approach is confounded by the plural effect of acquired mutations and by the presence of mutations that are either silent or do not contribute to thermostability. Despite several studies understanding the structural basis of thermostability has proven elusive and till date, there are no well-defined rules to stabilize a protein at high temperatures. Strategies that enable us to evolve enzymes under defined conditions and controlled physical stresses can help isolate protein variants with changes directly affecting the property of interest, for example, thermostability. These primary sequence variations can then be precisely mapped onto the structure. In vitro evolution is one such strategy, which attempts to simulate the natural evolution in vitro in generation of variants by error prone replicative processes and screening, for variants with the desired property.

Chapter 1

General aspects of protein thermostability and the structural features that contribute to protein thermostability are discussed. The 'directed evolution' method for engineering proteins and its application to the study of protein
thermostability are discussed. The structure, specificity, mechanism of action and applications of lipases, in general, and the *Bacillus subtilis* lipase, in particular, are discussed.

**Chapter 2**

The stability of the *B. subtilis* lipase in guanidinium chloride (GdmCl) has been studied. The lipase, lipA, from *B. subtilis* is a "lidless" lipase that does not show interfacial activation. Due to exposure of the active site to solvent, the lipase tends to aggregate. We have investigated the solution properties and unfolding of the lipase in GdmCl in order to understand its aggregation behavior and stability. Dynamic light scattering (DLS), near and far-UV circular dichroism, activity and intrinsic fluorescence of lipase suggest that the protein undergoes unfolding between 1 M and 2 M GdmCl. The polarity sensitive dye, 1,1',-bis-(4anilino)naphthalene-5,5'-disulfonic acid (bis-ANS), a probe for hydrophobic pockets, binds cooperatively to the native lipase. An intermediate populated in 1.75 M GdmCl that strongly binds bis-ANS was identified. Tendency of the native protein to aggregate in solution and specific binding to bis-ANS confirms that the lipase has exposed hydrophobic pockets and this surface hydrophobicity strongly influences the unfolding pathway of the lipase in GdmCl.

**Chapter 3**

To understand thermostability we employed the method of *in vitro* evolution to generate variants of the mesophilic lipase from *B. subtilis* showing "graded" thermostability. The lipase gene was randomly mutagenized and the clones expressing the mutant proteins were screened for increased thermostability of the lipase over the wild-type. The first generation yielded a clone, 1-1E5, which had a half-life of 25 min at 55 °C. This clone was used to parent the second generation. Two clones obtained in the second generation were recombined by restriction digestion-ligation and site-directed mutagenesis to yield two variants
-3B1 and 4B1- with half-lives 100 and 270-fold higher than that of the wild type protein, respectively. 4B1 has three mutations- N166Y, A132D and L114P. The amino acid changes conferring thermostability to the lipase were mapped on the crystal structure to find the structural basis for thermostability. It was found that the L114P mutation occurs near a very flexible loop of the protein and may be stabilizing the loop in the mutant. The A132D change replaces a solvent-exposed hydrophobic alanine near the active site with ionic aspartic acid. The Y166 residue in 4B1 might be stabilizing a neighbouring loop by forming a hydrogen bond with the backbone carbonyl of Leu160.

Chapter 4
The pH-dependent thermostability of the lipase was studied in the pH range 4-10. The B. subtilis lipase shows striking pH-dependent thermostability. The lipase was found to be very thermostable at lower pH (pH 4) and its thermostability decreased with increasing pH. The structure of the lipase at various pH's was probed by fluorescence and circular dichroism. Intrinsic fluorescence measurements and circular dichroism showed that the lipase, at different pH's, existed in different structural forms with altered tertiary structures but the same secondary structure content. The isothermal urea melts of the protein carried out at room temperature at various pH's showed that the lipase was conformationally more stable at pH 10 than at 4. This is in contrast to our observation that the lipase is more thermostable at lower pH's than at higher pH's and implies that the lipase at high pH undergoes temperature induced irreversible changes.

Chapter 5
Substrates insoluble in water are often presented to enzymes in micellized form. We observed anomalous increases in activities of lipases, with p-nitrophenyl oleate (PNPO)- Triton X-100 mixed micelles as the substrate system, in the presence of the protein denaturant GdmCl. However, with the
water-soluble substrate, p-nitrophenyl acetate (PNPA), the activity of the lipases decreased with increasing concentrations of GdmCI. Since chaotropes are known to affect micellar systems, we investigated the effects of GdmCI on the structure of the micelles and on the reactivity of PNPO present in mixed micellar form with Triton X-100. GdmCI does not affect the non-enzymatic hydrolysis rate of the water-soluble substrate PNPA substantially, however the hydrolysis of PNPO is enhanced 74-fold on increasing GdmCI concentration from 0 M to 6 M. This is accompanied by a 50% decrease in hydrodynamic radius and 25 times increase in critical micellar concentration (CMC). Polarity measurements and quenching studies with various analogues of anthroyloxy stearic acid incorporated into the Triton X-100 micelles indicated that in the presence of GdmCI the polarity extends deeper into the micelle. Quenching with the non-polar quencher, dimethylaniline (DMA), decreases in the presence of GdmCI due to altered partitioning of quencher. Increased quenching with the polar quencher, iodide, confirms increased water penetration in presence of GdmCI. Polarization of anthroyloxy moiety in GdmCI reported a progressively restrictive environment for probe mobility. Non-ionic micelles such as Triton X-100 do not possess intrinsic ability to catalyse hydrolysis reactions similar to ionic micelles; however, the present investigation demonstrates that perturbation of non-ionic micelle could lead to catalytic ability.